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Characterization of Genetic Diversity of *Bacillus anthracis* in France by Using High-Resolution Melting Assays and Multilocus Variable-Number Tandem-Repeat Analysis▽†

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Using high-resolution melting (HRM) analysis, we developed a cost-effective method to genotype a set of 13 phylogenetically informative single-nucleotide polymorphisms (SNPs) within the genome of *Bacillus anthracis*. SNP discrimination assays were performed in monoplex or duplex and applied to 100 *B. anthracis* isolates collected in France from 1953 to 2009 and a few reference strains. HRM provided a reliable and cheap alternative to subtype *B. anthracis* into one of the 12 major sublineages or subgroups. All strains could be correctly positioned on the canonical SNP (canSNP) phylogenetic tree, except the divergent Pasteur vaccine strain ATCC 4229. We detected the cooccurrence of three canSNP subgroups in France. The dominant B.Br.CNEVA sublineage was found to be prevalent in the Alps, the Pyrenees, the Auvergne region, and the Saône-et-Loire department. Strains affiliated with the A.Br.008/009 subgroup were observed throughout most of the country. The minor A.Br.001/002 subgroup was restricted to northeastern France. Multiple-locus variable-number tandem-repeat analysis using 24 markers further resolved French strains into 60 unique profiles and identified some regional patterns. Diversity found within the A.Br.008/009 and B.Br.CNEVA subgroups suggests that these represent old, ecologically established clades in France. Phylogenetic relationships with strains from other parts of the world are discussed.

Bacillus anthracis, the etiological agent of anthrax, is a spore-forming, Gram-positive bacterium belonging to the *Bacillus cereus* group. In the environment, *B. anthracis* primarily exists as quiescent spores that can persist for long periods of time in soil (35). Mammals—mainly wild and domesticated herbivores—are its natural hosts. Transmission to animals typically occurs through the gastrointestinal tract. Ruminants become infected by ingestion of soilborne spores while grazing. When inside the host, spores are phagocytized by macrophages and are carried to the lymph nodes, where they germinate and yield toxin-producing capsulated bacilli which rapidly multiply (21). The bacteria then enter the bloodstream and eventually cause septicemia. Septicemia and subsequent toxemia can rapidly lead to host death. The disease is acquired primarily through contact with infected animal products and can be transmitted to humans via the gastrointestinal, cutaneous, or respiratory routes (22).

B. anthracis is considered to be an evolutionarily young species, as suggested by its extremely low genetic variability (11, 12, 25). It is one of the most monomorphic bacterial pathogen species known, and its evolution is strictly clonal. Molecular typing techniques used to differentiate between *B. anthracis* strains therefore require high discriminatory power. Tandem-repeat polymorphisms, including minisatellites, microsatellites, single-nucleotide repeat (SNR), and single-nucleotide polymorphism (SNP), identified via whole genome sequence analyses have proven most successful in discriminating among *B. anthracis* strains (12–15, 17, 18, 24–26, 34).

Compared with the majority of polymorphic tandem-repeat markers, SNPs exhibit an extremely low mutation rate that makes them very valuable for broadly defining major phylogenetic divisions (13, 25). Using a large number of SNPs scattered throughout the whole genome, the phylogenetic relationships among *B. anthracis* isolates (25, 30) have been established. Researchers have further demonstrated that a small number of canonical SNPs (canSNPs), representative of specific branches and nodes in the *B. anthracis* tree, can be used to accurately define the major clades (13, 36). This leads to a genotyping method that uses a set of 13 strategically placed canSNPs to subdivide *B. anthracis* isolates into three recognized major lineages (A, B, and C), with further subdivision into 12 clonal sublineages or subgroups (36). This method had been applied to analyze several collections of *B. anthracis* strains of worldwide origin (2, 7, 23, 26, 33, 36). However, as it is based on TaqMan minor groove binding allelic discrimination assays, the current method incurs a high cost per studied marker. canSNP interrogation thus would greatly benefit from development of inexpensive alternative assays to increase access for research laboratories to these important phylogenetic markers.

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TABLE 1. Primer sequences

canSNP	Name	Forward primer (5′→3′)	Reverse primer (5′→3′)	Product size (bp)	Concn (μM) in duplex	Pair no.
A.Br.001	BA1A	GTGGTAAGGCAAGCGGAAC	ACGGTTTCCCTTTATCATCG	76	0.2	1
A.Br.002	BA2	GCAGAAGGAGCAAGTAATGTTATAGGT	CCTAAAATCGATAAAGCGACTGC	62	0.15	2
A.Br.003	BA3	AAAGGAATTTAGATTTTCGTGTCG	ATAAAAACCTCCTTTTTCTACCTCA	58	0.2	3
A.Br.004	BA4	ATCGCCGTCATACTTTGGAA	GGAATTGGTGGAGCTATGGA	53	0.15	3
A.Br.006	BA5	GCGTTTTTAAGTTCATCATACCC	ATGTTGTTGATCATTCCATCG	54	0.2	4
A.Br.007	BA6	TTACAAGGTGGTAGTATTCGAGCTG	TTGGTAACGAGACGATAAACTGAA	67	0.2	4
A.Br.008	BA7	CCAAACGGTGAAAAAGTTACAAA	GCAACTACGCTATACGTTTTAGATGG	80	0.2	5
A.Br.009	BA8	AATCGGCCACTGTTTTTGAAC	AGGTATATTAACTGCGGATGATGC	55	0.25	5
B.Br.001	BA9	GCACGGTCATAAAAGAAATCG	TGTTCAAAAGGTTCGGATATGA	75	0.2	2
B.Br.002	BA10	GCACCTTCTGTGTTCGTTGTT	TTCACCGAATGGAGGAGAAG	68	0.15	1
B.Br.003	BA11	ATTCGCATAGAAGCAGATGAGC	TCAAGTTCATAACGAACCATAACG	59	0.2	6
B.Br.004	BA12	TGCTTGGGTAACCTTCTTTACTT	AGAATAAAATGAAGATAATGACAAACG	62	0.3	6
A/B.Br.001	BA13	ATTCCAATCGCTGCACTCTT	CCCCGATAATTTTCACAAAGC	59		

High-resolution melting (HRM) analysis is a recent post-PCR technique that can detect sequence variations without the need for sequence-specific probes. HRM techniques can determine with high precision the melt profile of PCR products using a new generation of double-stranded DNA binding dyes and accurate fluorescence data acquisition over small temperature increments (as low as 0.01°C). The information in HRM analysis is contained in the shape of the melting curve rather than just the calculated melting temperature (T_m), therefore increasing the potential resolving power of this approach. Based on its ease of use, simplicity, flexibility, low cost, nondestructive nature, high sensitivity, and specificity, HRM analysis is quickly becoming the tool of choice to screen patients for pathogenic variants (37). It has also been employed for identification of various microorganisms, including bacteria (3, 10).

We report the development of an HRM-based SNP interrogation method that enables cost-effective genotyping of *B. anthracis* strains. Thirteen monoplex and six duplex HRM assays were designed to score the 13 published canSNPs. This method was used to determine the current phylogenetic position of 100 *B. anthracis* strains collected in France. Relationships among French isolates were further resolved using multiple-locus variable-number tandem-repeat (VNTR) analysis on 24 loci (MLVA24), and the congruence between the two methods was measured.

MATERIALS AND METHODS

Bacterial strains, growth, and biosafety procedures. The *B. anthracis* strains used as references for the HRM analysis were ATCC 4229, ATCC 14578, 17 JB, Sterne (CIP 77.2), strain Pasteur II (CIP 74.12), CIP A211, CIP A204, CIP 53.169, CNEVA 9066, and IEMVT 89 from the Institut d'Elevage et de Médecine Vétérinaire des pays Tropicaux. All French isolates were initially confirmed as *B. anthracis* based on bacteriology and PCR (29). These isolates included field strains collected from bovine, human, and environmental origin in France from 1953 to 2009. Strains were grown on horse blood agar petri dishes at 37°C.

Preparation of DNA samples. Bacterial colonies were taken from 24-h-old blood agar plates and mixed with 400 μl of water buffer. Suspensions were incubated at 100°C in boiling water for 20 min, cooled down for 10 min at 4°C, and centrifuged for 10 min at maximum speed. Viability testing was systematically performed to assess the complete removal of live forms of *B. anthracis* from DNA so that subsequent PCR testing could be carried out safely at lower levels of biocontainment. Briefly, 200-μl aliquots of each DNA preparation, including the cell pellets, were spread on horse blood agar petri dishes and grown at 37°C for 18 to 24 h. DNA used for MLVA was further purified using the High Pure PCR template preparation kit (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's recommendations, with final DNA elution in 200 μl.

HRM assays. The genomic positions of the 13 canSNPs can be found in supplemental Tables 4 and 5 in Van Ert et al. (36). Using Primer 3 Software (31), HRM primers were designed to amplify small amplicons (Table 1). Amplification was performed on the LightCycler 480 system using the LightCycler 480 high-resolution melting master mix (Roche Diagnostics). The reaction mixture consisted of 0.2 μM each primer, 1× LightCycler 480 HRM master mix, and 3 mM $MgCl_2$ in a 10-μl volume. Primer concentrations ranged from 0.15 μM to 0.3 μM in duplex reactions, as specified in Table 1. PCR amplifications were performed using about 5 ng of genomic DNA. The following parameters were used: 10 min at 95°C followed by 40 cycles consisting of 10 s at 95°C, 10 s at 58°C, and 10 s at 72°C. Samples were then heated to 95°C for 1 min, cooled to 50°C for 1 min, and heated from 65°C to 90°C at a rate of 1°C/s with 25 acquisitions/1°C. HRM data were analyzed with the LightCycler 480 gene scanning software (version 1.5.0) using non-temperature-shifted normalization curves ($t = 0$). The sensitivity parameter, which influences the stringency with which melting profiles are classified into different groups, was empirically adjusted so that all samples sharing the same allele were called "same" by the software and the remaining ones were denoted as different. The T_m of each amplicon was calculated using the companion T_m calling software (Roche Diagnostics).

MLVA. Twenty-four loci identified in previous studies (17, 18) were used, and MLVA was performed as described previously (18). Briefly, all 24 VNTR markers were amplified in four multiplex PCRs. Five to 10 ng DNA was used as the template in a final volume of 15 μl containing 1× PCR Roche reaction buffer, 0.2 mM deoxynucleoside triphosphates (dNTPs), the appropriate concentrations of each primer, and 1 U *Taq* polymerase (Roche Diagnostics). The labeled PCR products were diluted and added to a mix containing 40 μl of sample loading solution (Beckmann Coulter) and 0.5 μl of size marker (Bioventures or Beckman-Coulter). Samples were separated by electrophoresis in a CEQ separation gel (linear polyacrylamide [LPA I]) on a CEQ 8000 automatic DNA analysis system (Beckman-Coulter). The electropherograms were analyzed by the CEQ fragment analysis system software to determine the length of each fragment in reference to the size marker. All data produced were managed using BioNumerics software version 6.5 (Applied Maths, Sint-Martens-Latem, Belgium). Clustering was done using a graphing algorithm called a minimum spanning tree (MST). The priority rule for constructing MSTs was set so that the type that had the highest number of single-locus variants (SLVs) would be linked first. A cutoff value for a maximum difference of 2 out of 10 VNTRs was applied to define a cluster in the MST method.

Sequencing. DNA sequences of some canSNP regions from *B. anthracis* strains ATCC 4229 and IEMVT 89 were determined. PCR fragments of about 200 bp centered on canSNPs were amplified using 1 U of the GoTaq DNA polymerase (Promega). PCRs were carried out with a GeneAmp PCR System 9700 thermocycler (Applied Biosystems). The reaction conditions were 94°C for 10 min followed by 45 cycles of 94°C for 30s, 55°C for 30 s, and 72°C for 30 s. A final extension step of 72°C for 10 min was performed. Each amplicon generated was purified on the QIAquick purification kit (Qiagen) according to the manufacturer's instructions and sequenced by Eurofins MWG Operon (Ebersberg, Germany).

TABLE 2. Melting temperatures and SNP alleles determined for six reference strains using HRM

canSNP	Name	T_m (°C) (SNP allele)[a]					
		CNEVA9066	ATCC14578 (Vollum)	CIP 77.2 (Sterne)	CIP 74.12 (Pasteur II)	ATCC4229	IEMVT 89
A.Br.001	BA1A	75.92 (T)	76.09 (T)	76.02 (T)	76.10 (T)	**78.18** (T)	76.14 (T)
A.Br.002	BA2	79.19 (G)	79.22 (G)	78.32 (A)	79.31(G)	**–** (G)	79.14 (G)
A.Br.003	BA3	72.62 (A)	73.03 (A)	73.84 (G)	73.01 (A)	72.66 (A)	72.86 (A)
A.Br.004	BA4	77.55 (T)	77.45 (T)	78.34 (C)	77.52 (T)	**76.93** (T)	76.94 (T)
A.Br.006	BA5	78.19 (C)	77.41 (A)	77.24 (A)	77.38 (A)	**77.48** (A)	77.23 (A)
A.Br.007	BA6	74.67 (T)	75.66 (C)	74.65 (T)	74.84 (T)	**77.02**	74.56 (T)
A.Br.008	BA7	75.20 (T)	75.40 (T)	75.11 (T)	76.14 (G)	**74.56** (T)	75.11 (T)
A.Br.009	BA8	78.17 (A)	78.23 (A)	78.14 (A)	78.25 (A)	78.09 (A)	78.05 (A)
B.Br.001	BA9	74.95 (T)	75.08 (T)	75.03 (T)	75.00 (T)	**75.96 (A)**	74.85 (T)
B.Br.002	BA10	79.17 (G)	79.41 (G)	79.17 (G)	79.22 (G)	**78.69** (G)	79.10 (G)
B.Br.003	BA11	75.91 (A)	76.74 (G)	76.80 (G)	76.73 (G)	**–** (G)	76.53 (G)
B.Br.004	BA12	74.07(C)	73.41 (T)	73.16 (T)	72.97 (T)	**72.50** (T)	**71.95 (t**T)
A/B.Br.001	BA13	75.58 (A)	75.74 (A)	75.69 (A)	75.66 (A)	76.26 (G)	75.55 (A)
Subgroup		B.Br.CNEVA	A.Br.Vollum	A.Br.001/002	A.Br.008/009	**new A.Br.**	A.Br.005/006

[a] –, no HRM amplification; CIP, collection number of Pasteur Institute. Unexpected T_m values and nucleotide polymorphisms are indicated in bold.

Nucleotide sequence accession numbers. EMBL accession numbers are HE598698 to HE598707 (strain ATCC 4229) and HE598697 (strain IEMVT89).

RESULTS

HRM assay design and validation. Thirteen SNP discrimination assays were designed to screen by HRM the set of canSNPs identified and selected by Van Ert et al. (36). Primers matching sequences close to each canSNP were chosen to maximize the differences in melting temperatures that the SNP confers (Table 1). Assays were evaluated on a panel of 10 reference strains, including five known canSNP genotypes. All strains except the Pasteur vaccine strain ATCC 4229 gave amplicons producing a single melting peak for the 13 monoplex assays. The two expected alternate alleles exhibited distinct melting curves and T_m values. On average, differences in T_m values of about 1°C were observed between the two allelic states, with calculated T_m values ranging from 71.95°C to 79.3°C (Table 2 and Fig. 1). As illustrated in Fig. 1, differences in the shapes of the melting curves allowed clear separation and unambiguous grouping of each alternate allele by the LightCycler 480 gene scanning software. HRM was found to be a robust and reproducible method to score canSNP genotypes.

The HRM analysis also highlighted some discordant results that suggest unexpected sequence variability at several canSNP loci. Discrepancies between observed and expected HRM results were demonstrated for the IEMVT 89 and ATCC 4229 strains (Fig. 1 and Table 2). These two strains were sequenced to identify new polymorphisms and ultimately classify both samples.

The IEMVT 89 strain, which was the only specimen isolated from Africa in the *B. anthracis* reference panel, was scored as a third distinct group by the B.Br.004 HRM assay. The amplicon displayed a T_m value of 71.95°C (compared to about 73°C and 74°C for the expected canSNP T and C alleles, respectively) and a modified melting curve shape. Sequencing revealed a second mutation contiguous to the canSNP position (T allele). This additional substitution of a C with a T can explain the observed discrepancy. Taken together, these results show that the IEMVT 89 strain shares the canSNP characteristics of the A.Br.005/006 canSNP subgroup but can be distin-

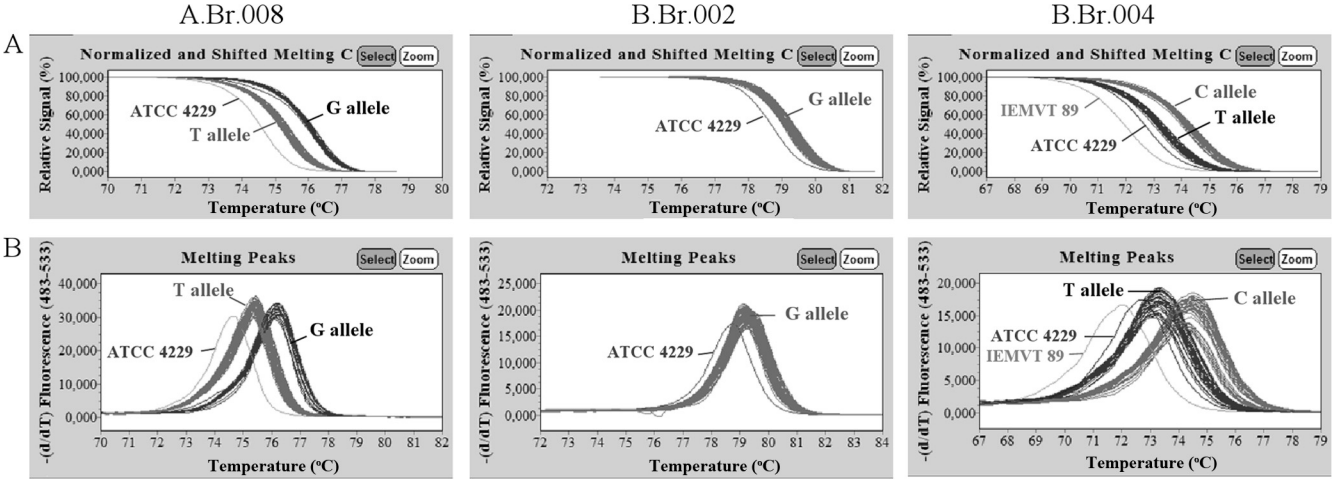

FIG. 1. HRM analysis of three canSNPs using monoplex assays on 90 *B. anthracis* strains. (A) Normalized melting curve; (B) negative derivative of fluorescence with respect to temperature. Data and plots were produced by the LightCycler 480 system using gene scanning software.

FIG. 2. Location of *B. anthracis* canSNP genotypes in France. Map shows actual collection sites where isolates of known origin were obtained for this study. Strains affiliated with the B.Br.CNEVA, A.Br.001/002, and A.Br.008/009 subgroups are indicated, respectively, by dark circles, stars, or gray squares. Size is proportional to the number of isolates.

guished from the other strains in this subgroup by its unique sequence at the B.Br.004 canSNP locus.

In contrast, the ATCC 4229 Pasteur strain, a heat-attenuated, toxin-negative strain, could not be placed into any of the 12 subgroups previously defined by the *B. anthracis* canSNP-derived phylogenetic tree. The strain failed to amplify at two canSNP loci (A.BR.002 and B.Br.003) and was denoted as a distinct genotype at eight other canSNPs. In addition, amplification was of lower efficiency than that of the other strains. Sequencing revealed numerous mutations within the corresponding PCR products (including primer annealing sites) that were consistent with the discrepancies observed in PCR efficiency, melting curve shape, or T_m values. Interestingly, the ATCC 4229 strain was also PCR negative when screening for another *B. anthracis*-specific SNP used to confirm *B. anthracis* identity, i.e., the nonsense mutation at nucleotide position 640 of the *plcR* gene (4, 16). Sequencing indicated a 12-nucleotide deletion at this position (data not shown), further highlighting the unique character of this particular strain.

canSNP typing of French isolates. HRM assays were then used to examine the phylogenetic position and genetic diversity of French *B. anthracis* isolates. One hundred field strains representative of anthrax activity in France since 1953 were selected for this study. Most of them were collected during animal anthrax outbreaks that have occurred over the past 20 years (19, 20). The CNEVA9066 and Sterne 77.2 strains were used as internal controls for run-to-run normalization. Melting profiles denoted as "same" or "different" by the software were scored into one of the two expected allelic states. No discrepancies were observed. All strains were unambiguously classified into three of the 12 phylogenetic subgroups identified worldwide, A.Br.001/002, A.Br.008/009, and B.Br.CNEVA.

Localization of these three subgroups in France is shown in Fig. 2. The majority of strains (54%) were part of the B.Br.CNEVA sublineage. This sublineage was prevalent in four areas, i.e., the Alps, the Pyrenees, the Auvergne region, and the Saône-et-Loire department. About one-third of French strains (30%) belong to the A.Br.008/009 subgroup. Strains affiliated with A.Br.008/009 were isolated throughout the country. The remaining strains (16%) were from the A.Br.001/002 subgroup. Most of these strains (13 out of 16) were collected during 17 clustered animal outbreaks occurring in the summer of 2008 in the Doubs department, near Switzerland (19). The other strains are older isolates from human cases.

Multiplexing assays. To increase the analysis throughput, the overall HRM genotyping assay was converted into six duplex PCRs (Table 1) and applied to 40 strains representative of the diversity (genetic and geographic) found in France. Primer concentrations were adjusted so as to amplify two canSNPs with similar efficiency and to yield melting peaks of equivalent height for both amplicons. As illustrated in Fig. 3, the HRM method was still able to successfully resolve the different variants in duplex reactions. Although correct grouping by the

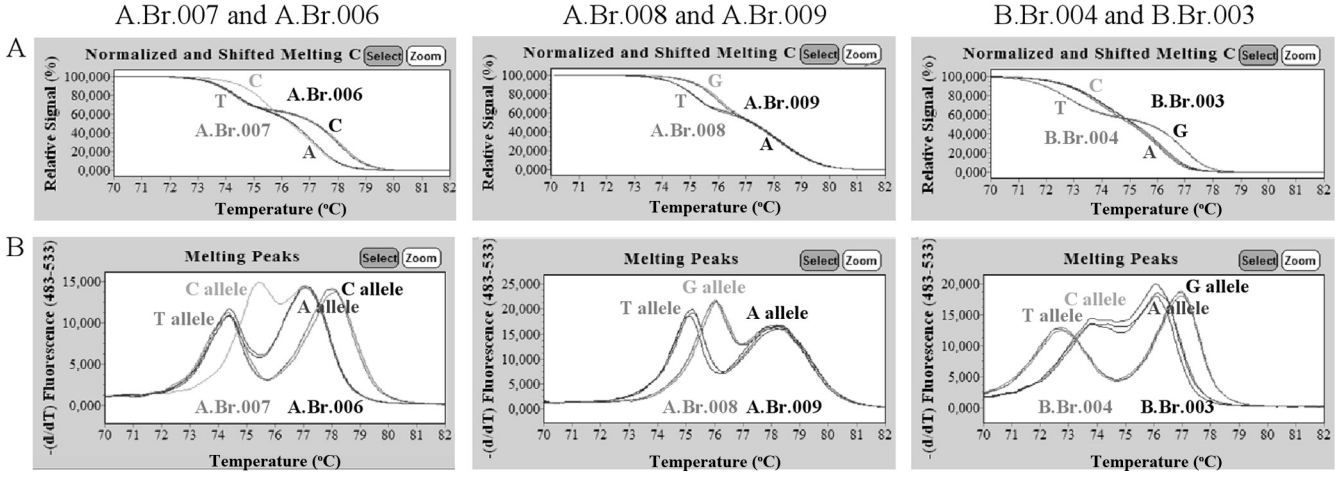

FIG. 3. HRM analysis of six canSNPs using duplex assays on six *B. anthracis* strains. (A) Normalized melting curve; (B) negative derivative of fluorescence with respect to temperature. Data and plots were produced by the LightCycler 480 system using gene scanning software.

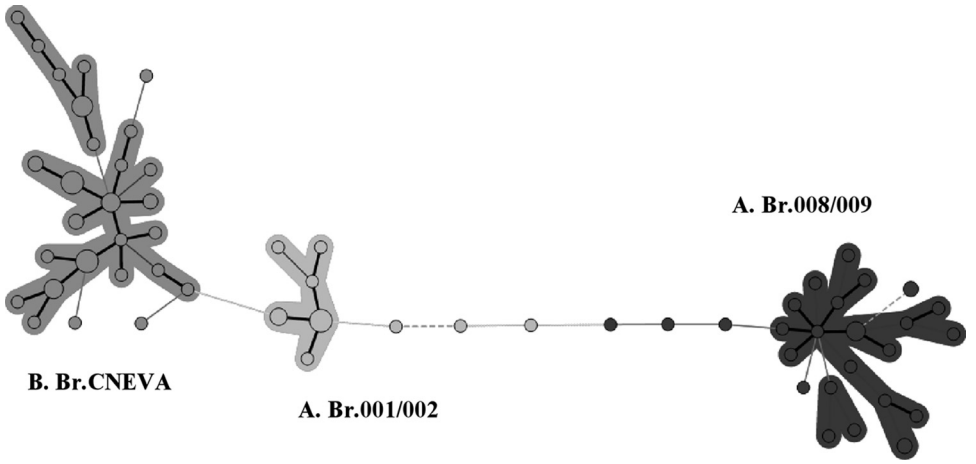

FIG. 4. Minimum spanning tree of 95 *B. anthracis* isolates based on categorical analysis of 24 VNTRs. Each circle represents a unique genotype. The diameter of each circle varies according to the number of isolates having the same genotype. Genotypes connected by a shaded background differ by a maximum of two VNTR markers. Thick and regular connecting lines represent a difference of one and two markers, respectively; thin interrupted lines represent a difference of three markers; thin dashed lines represent four or more differences. The length of each branch is also proportional to the number of differences.

gene scanning software may need empirical adjustments of the sensitivity parameter to compensate for variability in PCR efficiency, HRM results were reproducible and data were consistent with the 13 monoplex assays. All samples were unambiguously subtyped into one of the 12 canSNP genotypes.

MLVA of French isolates. Twenty-four VNTR markers from the MLVA25 system developed by Lista et al. (18) were used to further subtype most of the French strains. The whole MLVA data set revealed at least 60 different genotypes. Within canSNP subgroups, strains were, respectively, resolved into nine (A.Br.001/002 subgroup, $n = 16$ strains), 24 (A.Br.008/009, $n = 30$ strains), and 27 (B.Br.CNEVA, $n = 50$ strains) unique genotypes (see the supplemental material). Genetic relationships among French *B. anthracis* isolates are graphically presented in Fig. 4 using a minimum spanning tree. As expected, canSNP subgroups were quite dissimilar and loosely connected to each other. More specifically, they differed in the number of repeat copies at the Bams01 marker, with a unique allele size of 16 repeats for A.Br.001/002, 13 repeats for A.Br.008/009, and 14 repeats for B.Br.CNEVA. In contrast, close genetic relationships were detected between most genotypes of a given canSNP subgroup, and regional strain patterns were observed within the B.Br.CNEVA sublineage using the Bams34, Bams22, or Bams51 markers. For instance, isolates from the Pyrenees and Aube departments were characterized by 8 repeat copies (versus 6 repeats for the other strains) for marker Bams51 and by 9 repeat copies for marker Bams34 (versus 11 for strains collected in Sâone-et-Loire or 13 repeats for strains from Auvergne and the Alps). Isolates from the Alps had 13 repeat copies (versus 15 repeats for the other strains) for marker Bams22.

DISCUSSION

The analysis of a small set of canonical SNPs is a fast way to determine the major clonal sublineages of *B. anthracis*. Cost is an important issue for all diagnostic and phylogenetic assays. The setup described by Van Ert et al. (36) requires the use of 26 sequence-specific TaqMan minor groove binder (MGB) probes. This setup incurs high costs in laboratories where only a few strains have to be typed per year. The development of cheaper alternatives for interrogating canSNPs would increase access to these important markers for a larger number of laboratories. HRM is an attractive method, as it is faster, simpler, and less expensive than alternative approaches requiring separations or labeled probes (28, 39). HRM is a single-step, closed-tube assay that has high discriminatory power. The HRM assay requires approximately 1 h for a run, including the follow-up data analysis, on the LightCycler 480 instrument and can be performed in reaction volumes of less than 10 μl, reducing *de facto* the cost per analysis.

Although it is highly recommended to standardize the quality and amount of DNA templates to minimize reaction-to-reaction variability in HRM assays, these parameters were not found to be extraordinarily critical for distinguishing homozygotic variants unless insufficient amplification had occurred. Any sample showing late amplification (threshold cycle [C_T] value over 36) was discarded to avoid misidentified classification of samples. Two reference strains with distinct allelic states (CNEVA9066 and Sterne 77.2) were also included for run-to-run normalization to ensure that the correct allele is called. Indeed, although the relative temperature calibration is extremely accurate, absolute temperature calibration can vary between runs (by up to 0.5°C). The developed assay successfully differentiated between the different *B. anthracis* sublineages in our panel of strains. We thus conclude that HRM is a fast and reliable diagnostic technique for bacterial genotyping. Finally, the method was demonstrated to be amenable to some multiplexing. The monoplex assays were converted into a duplex format without compromising efficiency or accuracy. Both formats provided reproducible melting curves and consistent SNP genotyping data.

Besides capacity for SNP discrimination, the HRM technique can also be used for gene scanning and detection of new polymorphisms. Any amplicon containing one or more unex-

pected sequence variations would have a different HRM profile compared to those of controls, although SNPs involving C-to-G or A-to-T substitutions are difficult to distinguish. As a result, any sample with aberrant curve shapes requires further analysis by sequencing to confirm and identify the new mutation(s). We reported conflicting data in two strains. The Pasteur vaccine strain ATCC 4229 gave discordant results for 10 canSNP loci and could not be placed into any sublineages of the original canSNP tree defined by Van Ert et al. (36). The strain did not carry the nonsense mutation in *plcR*, a characteristic that differentiates the *B. anthracis* lineage from the rest of the *B. cereus* group (16). Shotgun sequencing and comparison to other genomes had recently positioned this strain into the major A lineage of *B. anthracis* but on its own, specific branch point (32). Taken together, these data suggest that this peculiar strain may represent a borderline strain that should be classified as a new, additional subgroup in the current canSNP scheme (36). In contrast, the IEMVT 89 strain, which exhibited a single deviant melt profile, did share the expected canSNP characteristics of the A.Br.005/006 subgroup. One sequence variation adjacent to the B.Br.004 canSNP could explain the aberrant curve shape and T_m observed. Considering that the IEMVT 89 strain was the sole Central and West Africa isolate of our collection, we could not conclude whether this additional SNP represents a new informative canSNP marker (specific to strains from these regions) or just a single point mutation in a particular strain. Interestingly, it has been recently reported that *B. anthracis* strains isolated in Cameroon and Chad carry the same additional base difference just downstream of the canSNP B.Br.004 (27), suggesting that this particular substitution of a C with a T could be a new informative canSNP marker specific to strains from this part of the world. Examination of more African specimens is needed to confirm this hypothesis.

The present work establishes the first overall picture of the genetic diversity of *B. anthracis* in France and provides valuable data sets for future epidemiological or forensic studies. Three greatly differing canSNP genotypes cooccur in France. The majority of strains (54%) are affiliated with the B.Br.CNEVA sublineage. This lineage is found exclusively in Western and Central Europe (12, 36). Primarily reported from southern France (6), strains belonging to B.Br.CNEVA have also been recovered from northern Italy, Croatia, Slovakia, Switzerland, Germany, and Poland (7–9, 26, 36), where it represents autochthonous cases from cattle that died from anthrax. While previous MLVA8 analysis resolved only two different genotypes among 27 French isolates affiliated with the B2 cluster (6), MLVA24 resolved 27 distinct genotypes in this study. Spatial differences seen between MLVA profiles suggest a successful establishment and *in situ* differentiation of *B. anthracis* within regions. The sublineage is prevalent in four geographical areas: the Alps, the Pyrenees, Auvergne, and the Saône-et-Loire department. It may be of interest to note that these zones, close to the mountainous massifs of France, share similar land topologies, made of pastoral valleys. Our data, consistent with other MLVA studies reporting new additional genotypes among Italian, Polish, and Swiss isolates (7–9, 26), indicate that the B.Br.CNEVA sublineage may be relatively genetically diverse.

The second group (30% of strains) observed in France is A.Br.008/009, a widely dispersed subgroup spread across most of Europe and Asia. A.Br.008/009 is the most common type observed in Italy (5), some Eastern European countries (2, 36), and Central Asia (1, 23, 33). French isolates affiliated with the A.Br.008/009 subgroup were collected in various areas and times throughout the country and a particular spot in Bourgogne (in the Cotes-d'Or department) where recurrent outbreaks have occurred. Cooccurrence of this subgroup with the B.Br.CNEVA sublineage was observed in a single area, the Pyrenees. A complex pattern of 24 distinct genotypes was resolved by MLVA, suggesting that the A.Br.008/009 subgroup also has an extensive history in France.

We reported for the first time the presence of the A.Br.001/002 canSNP subgroup in France. A.Br.001/002 is found commonly in Eastern Asia and China, where it represents an old established clade (33). The strains found in Europe (36, 38) may represent old imported infections from Asia via animals or human activities, such as trading (33). A.Br.001/002 is a minor group (16%) in France that seems to be geographically restricted to the northeast. Most specimens were isolated in 2008 during an episode involving 17 clustered outbreaks, with many cases in Doubs (19). These strains were resolved in six MLVA24 profiles showing minor variations. They were only loosely connected to three older strains from the Pasteur Institute Collection that formed a separate cluster.

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